

LABORATORY SCIENCES

Combined Effect of Cyclosporine and Sirolimus on Improving the Longevity of Recombinant Adenovirus-Mediated Transgene Expression in the Retina

Wei-Yong Shen, MD; May C. Lai, PhD; John Neilby, MD; Nigel L. Barnett, PhD; Jie Liu, MD; Ian J. Constable, MD; Piroska E. Rakoczy, PhD

Objectives: To reevaluate the longevity and intracocular safety of recombinant adenovirus (rAd)-mediated gene delivery after subretinal injection, and to prolong transgene expression through the combination of 2 synergistic immunosuppressants.

Methods: An rAd vector carrying green fluorescent protein (GFP) gene was delivered subretinally in the rat eye. The GFP expression was monitored in real time by fundus fluorescent photography. Intracocular safety was examined by observation of changes of retinal pigmentation, cell infiltration in virus-contacted area, immunophenotyping for CD4⁺ and CD8⁺ cytotoxic T lymphocytes, and CD68⁺ macrophages, histologic findings, and dark-adapted electroretinography. Two synergistic immunosuppressants, cyclosporine and sirolimus, were used alone or in combination to prolong transgene expression by temporary immunosuppression.

Results: The GFP expression peaked on day 4, dramatically decreased on day 10, and was not detectable on day 14. The decreased GFP expression was coincident with cell infiltration in virus-contacted area. Immunostain-

ing showed that the infiltrating cells were CD4⁺ and CD8⁺ cytotoxic T lymphocytes and CD68⁺ macrophages. Clumped retinal pigmentation and decreased b wave of dark-adapted electroretinogram were observed at 3 to 4 weeks after injection. Histologic examination confirmed rAd-induced retinal degeneration. Transient immunosuppression by cyclosporine and sirolimus, either alone or in combination, improved transgene expression, with the combination being the most efficient. The combined immunosuppression attenuated but did not retard the rAd-induced retinal damage.

Conclusions: Transgene expression mediated by rAd after subretinal delivery is short-term and toxic to the retina. Combination of cyclosporine and sirolimus may act as an immunosuppressive adjunct to prolong rAd-mediated gene transfer.

Clinical Relevance: The intracocular safety of rAd should be carefully considered before clinical trials are performed.

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From the Centre for Ophthalmology and Visual Science, University of Western Australia, Perth (Drs Shen, Lai, Liu, Constable, and Rakoczy); Centre for Pathology and Medical Research, Queen Elizabeth II Medical Centre, Perth (Dr Neilby); and Vision, Touch & Hearing Research Center, Department of Physiology and Pharmacology, University of Queensland, Brisbane, Australia (Dr Barnett).

GENE THERAPY, which has the ability to produce high concentrations of therapeutic agents in situ for sustained periods, has the potential to change traditional treatments for human diseases. The eye is an ideal target for gene therapy because it is separated from other organs by the blood-retinal barrier; there are a limited number of cells confined to a small space, and transgene expression can be monitored optically without invasive interventions.^{1,2}

Recombinant adenovirus (rAd) is one of the most widely used viral vectors targeting both resting and proliferating cell types. In the eye, subretinal delivery of rAd almost exclusively transduces the retinal pigment epithelium (RPE) with high efficiency.^{3,4,5} The preferential transduction of RPE cells is potentially useful to develop

novel strategies for the treatment of retinal degenerations.^{6,7} It also seems realistic that delivery of neurotrophic factor genes to the RPE cells could attenuate the progress of retinitis pigmentosa.^{8,9} However, the longevity of rAd-mediated transgene expression in the eye dramatically varied from days to months in previous studies.^{2,4,7,10} The use of green fluorescent protein (GFP) as a reporter gene may allow us to noninvasively clarify this issue in living animals.

Host immune responses to E1 and E3 region deleted rAds currently present a general problem for long-term gene delivery. Cytotoxic T lymphocytes have been shown to mediate the destruction of rAd-transduced cells. Humoral response leads to the production of antibodies, inhibiting further delivery of rAd.^{10,11} Traditionally, the eye has been considered an im-

MATERIALS AND METHODS

GENERATION OF rAd

All manipulations regarding the rAd were performed in accordance with the institutional biosafety guidelines. The GFP was selected as a reporter gene because its expression can be monitored noninvasively in real time in the eye.¹³ The rAd, Ad.CMV.GFP, has an E1 and partial E3 region deletion, and a titer of 3.0×10^{10} plaque-forming units/mL was used in this study.

SUBRETINAL ADMINISTRATION OF Ad.CMV.GFP

All animal experiments adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Normal congenic pigmented and nonpigmented RCS/rdy rats 4 to 6 weeks of age were used in this experiment. A 2- μ L solution containing 6.0×10^7 plaque-forming units of virus was delivered into the subretinal space as described previously.²² Phosphate-buffered saline (PBS) was injected in the same way as a control. Successful administration into the subretinal space was confirmed by the appearance of a subretinal bleb under the operating microscope in nonpigmented eyes and a partial retinal detachment by indirect ophthalmoscopy in pigmented eyes.

IMMUNOSUPPRESSION BY CYCLOSPORINE IN COMBINATION WITH SIROLIMUS

For immunosuppression, cyclosporine (Novartis Pharmaceuticals Australia Pty Limited, North Ryde, Australia) was diluted with water for injection to yield a concentration of

10 mg/mL. Sirolimus (kindly provided by Wyeth-Ayerst Research, Monmouth Junction, NJ) was reconstituted in 0.2% carboxymethylcellulose to a final concentration of 2 mg/mL. The subretinally injected rats were divided into 4 groups: (1) vehicle, 0.2% carboxymethylcellulose ($n=9$); (2) cyclosporine, 10 mg/kg per day ($n=7$); (3) sirolimus, 2 mg/kg per day ($n=7$); and (4) combination of cyclosporine plus sirolimus, 10 and 2 mg/kg per day, respectively ($n=11$). Immunosuppression was initiated immediately after subretinal administration of Ad.CMV.GFP by intraperitoneal injection once a day for 2 weeks. Carboxymethylcellulose, the vehicle used to reconstitute sirolimus, was injected in the same way as a control. All animals were housed in rooms that were suitable for work with microorganisms and biological hazards under barrier conditions to prevent infection. Animals were weighed weekly for 5 weeks to monitor their general conditions under immunosuppression.

NONINVASIVE EVALUATION OF GFP EXPRESSION AND CLINICAL EXAMINATIONS

The GFP expression was observed by fundus fluorescent photography (FFP) at 4, 7, and 10 days, then weekly for 10 weeks, as reported previously.²³ The fluorescent signal was assessed by 2 independent observers (W.-Y.S. and M.C.L.) and graded on a scale of 0 to 5 that involved both the transduced area and GFP signal intensity. For area, a score of 5 indicated 80% to 100%; 4, 60% to 79%; 3, 40% to 59%; 2, 20% to 39%; 1, less than 19%; and 0, negative. For intensity, a score of 5 indicated very strong; 4, strong; 3, moderate; 2, weak; 1, very weak; and 0, negative. Fundus color photography was performed periodically in pigmented eyes, and fluorescein angiography was performed when GFP expression was not detectable.

immune-privileged site. However, the intraocular safety after subretinal delivery of rAd is controversial.^{12,14} Recent data indicate that both humoral and cellular immune responses are evoked by rAd directly delivered into the subretinal space.^{14,15} This raises the possibility that suppression of the host immune system may significantly improve the efficacy of transgene expression.

Cyclosporine has been shown to be effective in the treatment of immune-mediated ocular diseases. However, it is associated with renal, hepatic, and neurologic toxic effects that often limit its use in a high dose or for a long-term application.^{16,17} Sirolimus, a hydrophobic 31-membered macrocyclic lactone ($C_{51}H_{79}NO_{13}$), has been demonstrated to inhibit a variety of experimental autoimmune diseases, retard neoplastic growth, and prolong organ allograft survival.^{18,19} There is accumulating evidence that low doses of cyclosporine and sirolimus have synergistic effects on immunosuppression that may significantly reduce the risk of in vivo toxic effects.^{20,21}

For all of these reasons, in this study we aimed to reevaluate the longevity and intraocular safety of rAd-mediated gene delivery in real time after subretinal injection, and to prolong transgene expression by transient immunosuppression via combination of cyclosporine and sirolimus.

RESULTS

TRANSGENE EXPRESSION AND CHANGES OF RETINAL PIGMENTATION

After subretinal injection of Ad.CMV.GFP, the retina images of nonpigmented eyes appeared to be normal when observed by fundus color photography (Figure 1A). At 4 days after injection, strong GFP expression was detected in all eyes, although there was some variability in the size of the fluorescent area (Figure 1C). However, GFP expression rapidly declined to a basal level at day 10 (Figure 1E) and was not detectable at day 14 by FFP (Figure 1G).

Considering the difficulty of distinguishing penetration-induced retinal damage from rAd-induced toxic effects on histologic examination, fundus color photography was performed in pigmented eyes ($n=8$) to visualize the changes after rAd administration. The pattern of GFP expression in pigmented eyes was the same as that observed in nonpigmented eyes (Figures 1B, D). However, disturbed pigmentation was observed by 3 to 4 weeks after injection (Figure 1F). Fluorescein angiography further demonstrated an appearance of "RPE window de-

RPE CHOROID-SCLERAL FLAT MOUNT: PREPARATION AND IMMUNOHISTOCHEMISTRY

After FFP, 8 eyes were enucleated at days 4, 7, 10, and 14 (2 eyes at each time point) for RPE-choroid-scleral (R-C-S) preparation, and 4 eyes were enucleated and snap frozen in optimal cutting temperature compound at day 7 for immunohistochemistry. Four PBS-injected eyes were enucleated at 7 days as controls. The R-C-S whole mounts were examined by fluorescent microscopy to evaluate the association between GFP expression and local cellular infiltration. Frozen sections of 12 to 14 μ m thickness were produced for CD4⁺ and CD8⁺ cytotoxic T-lymphocyte immunostaining as described previously,¹¹ with the use of monoclonal antibodies against rat CD4⁺ (1:50), CD8⁺ (1:50) lymphocytes, and CD68⁺ (1:500) macrophages (Serotec Inc, Kidlington, Oxford, England).

ELECTRORETINOGRAPHY

Nine eyes received subretinal administration of Ad.CMV.GFP and were treated with vehicle (n=4) or cyclosporine plus sirolimus (n=5), and 6 eyes from age-matched untreated rats were assessed by electroretinography (ERG) at 4 weeks after injection. After anesthesia and pupil dilation, the animals were allowed to adapt to the dark for 30 minutes before the scotopic flash ERGs were performed. A platinum wire loop was placed on each cornea to act as the recording electrode, and a reference electrode was connected to the ear. Ground electrodes were attached to the animal's back. A xenon strobe light placed 0.5 m in front of the animal presented the flash stimulus at 0.25 Hz. Eight consecutive responses were amplified and averaged by means of a bioamplifier/data recorder (MacLab/2c; ADInstruments, Sydney, Australia) running Scope software (ADInstruments).

WHITE BLOOD CELL COUNTING AND SERUM BIOCHEMICAL ANALYSIS

By 5 weeks after injection, blood samples were taken from 20 rats under immunosuppression. From each animal, 0.5 mL of blood was collected in a heparinized tube for analysis of white blood cell count and differential count (Beckman Coulter, Sydney, Australia). Another 2.0 mL of blood was collected for analysis of glucose level and renal and liver function by standard procedures on an automated analyzer (Hitachi 747 Autoanalyser, Boehringer Mannheim, Sydney, Australia).

HISTOLOGIC EXAMINATION

Eyes were enucleated at 5 (n=22) and 10 (n=14) weeks after injection. After eye enucleation and blood sample collection, the liver and kidney were also harvested. The sampled tissues were fixed by 4% paraformaldehyde for paraffin embedding. Paraffin sections 5 to 6 μ m thick were processed for hematoxylin-eosin staining to evaluate the retinal morphologic characteristics, and sections from livers and kidneys were examined for the evidence of systemic toxic effects of immunosuppression.

STATISTICAL ANALYSIS

All results are expressed as mean \pm SD. Two unpaired groups were compared with the 2-sample t test. Analysis of variance was used to compare 3 or more groups. Differences were considered statistically significant at $P < .05$.

fect" with stunning exposure of the choroidal large vessels underneath the retina (Figure 1H). No vascular leakage was detected during fluorescein angiography. The retinal depigmentation was not observed in PBS-injected eyes (data not shown).

GFP EXPRESSION, LOCAL CELLULAR INFILTRATION, AND IDENTIFICATION

By examination of the R-C-S whole mounts, strong GFP expression was observed at 4 days after injection while few inflammatory cells were detected, and GFP was localized to the hexagonal RPE cells (Figure 2A, D). By 7 days, GFP expression was mostly detected in the peripheral part of the virus-injected area, where the RPE morphologic characteristics remained relatively normal. Infiltrating cells, however, were observed in the center, accompanied by a decline in GFP expression. The RPE cells became disorganized, resulting in the presence of "ghost" RPE cells (Figure 2B, E). The GFP expression was not detectable at day 14. Instead, a large amount of inflammatory cells accumulated in the virus-contacted area, and RPE elimination was observed (Figure 2C, F). Massive cell infiltration and RPE damage were not observed in PBS-injected eyes (data not shown).

By fluorescent microscopy of cryosections, cellular infiltration was observed in the subretinal space and neural retina from 7 days after injection (Figure 3A). Immunophenotyping showed that most of the infiltrating cells were CD8⁺ and CD4⁺ cytotoxic T lymphocytes (Figure 3B, C), and macrophages (CD68⁺) were also detected (Figure 3D). By 4 weeks after injection, cell infiltration was dramatically decreased, but retinal degeneration was observed in all rAd-injected eyes, demonstrated as reduced number or total loss of the outer nuclear layer and slightly affected inner nuclear layer (Figure 3E, F). Except for the occasional presence of macrophages (CD68⁺) at the site of injection, CD4⁺ and CD8⁺ cytotoxic T lymphocytes and retinal degeneration were not observed in PBS-injected eyes (data not shown).

PROLONGED TRANSGENE EXPRESSION BY IMMUNOSUPPRESSION

Monitored by real-time observation, the efficiency and longevity of GFP expression in vehicle-treated rats were similar to those observed in nontreated rats. The GFP expression was detected in all groups at 4 days after injection (Figure 4A-C and Figure 5). However, no fluorescent signal was detected in the vehicle-treated group at week

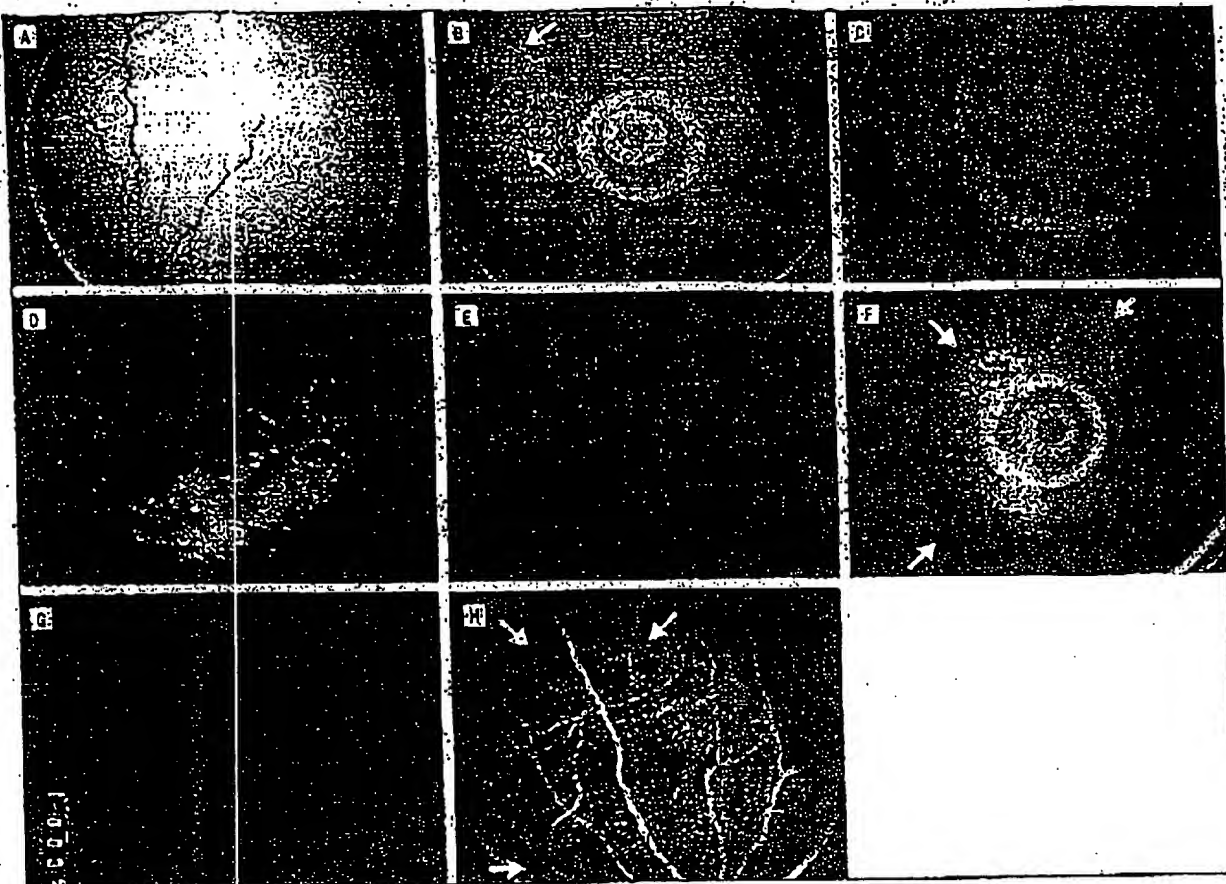


Figure 1. Green fluorescent protein expression and retinal images after subretinal injection of Ad.CMV.GFP at 4 days (A-D), 10 days (E), 2 weeks (G), and 4 weeks (F and H) after injection. A, C, and F are retinal images obtained from fundus color photography. C to E and G are images obtained from fundus fluorescent photography. H shows the disturbed retinal pigmentation and choroidal capillaries of F, demonstrated by fluorescein angiography. Arrows in B point to the route of needle penetration. Arrows in F and H indicate the areas with disturbed retinal pigmentation. Asterisks in B, F, and H indicate a site with progressive retinal degeneration (original magnification $\times 6$).

2 (Figure 5). At 4 weeks after injection, the percentage of eyes with GFP expression was 67%, 50%, and 9% in groups treated with cyclosporine plus sirolimus, cyclosporine, and sirolimus, respectively. Single drug application with sirolimus or cyclosporine prolonged GFP expression for up to 4 and 9 weeks, respectively. By 10 weeks after injection, 25% of the eyes in the cyclosporine plus sirolimus-treated group still showed GFP expression (Figure 5).

To compare the effect of different strategies on transgene expression, the levels of GFP expression were graded by semiquantification that involved both the area and intensity of fluorescent signal (Figure 6). Although cyclosporine and sirolimus, either alone or in combination, prolonged GFP expression, GFP expression still decreased with time at a slower rate during the first 2 weeks. Interestingly, a rebound GFP expression was observed at 1 week after withdrawal of immunosuppression (3 weeks after injection) in animals treated with cyclosporine plus sirolimus ($P=.02$) and cyclosporine ($P=.04$) but not by sirolimus ($P>.05$). At 3 and 4 weeks after injection, the levels of GFP expression in the sirolimus-treated group were significantly lower when compared with the cyclosporine plus sirolimus- and cyclo-

sporine-treated groups, respectively ($P<.01$). The GFP expression in cyclosporine plus sirolimus- and cyclosporine-treated groups were comparable for up to 5 weeks. However, from 6 to 9 weeks, the GFP levels in the cyclosporine-treated group were significantly lower than those observed in the cyclosporine plus sirolimus-treated group ($P<.05$). Only the cyclosporine plus sirolimus-treated group demonstrated GFP signal when observed by GFP at 10 weeks after injection.

ATTENUATION OF rAd-INDUCED RETINAL DAMAGE BY COMBINED IMMUNOSUPPRESSION

The combined immunosuppression by cyclosporine plus sirolimus attenuated but did not stop rAd-induced retinal damage (Figure 7). The ERG results are summarized in Table 1. By 4 weeks after injection, the implicit time of the a and b waves and the mean amplitudes of the a wave were not significantly different between groups. However, the mean amplitudes of the b wave significantly decreased in the group treated with the vehicle (0.2% carboxymethylcellulose) ($P=.03$) and slightly decreased in the cyclosporine plus sirolimus-treated

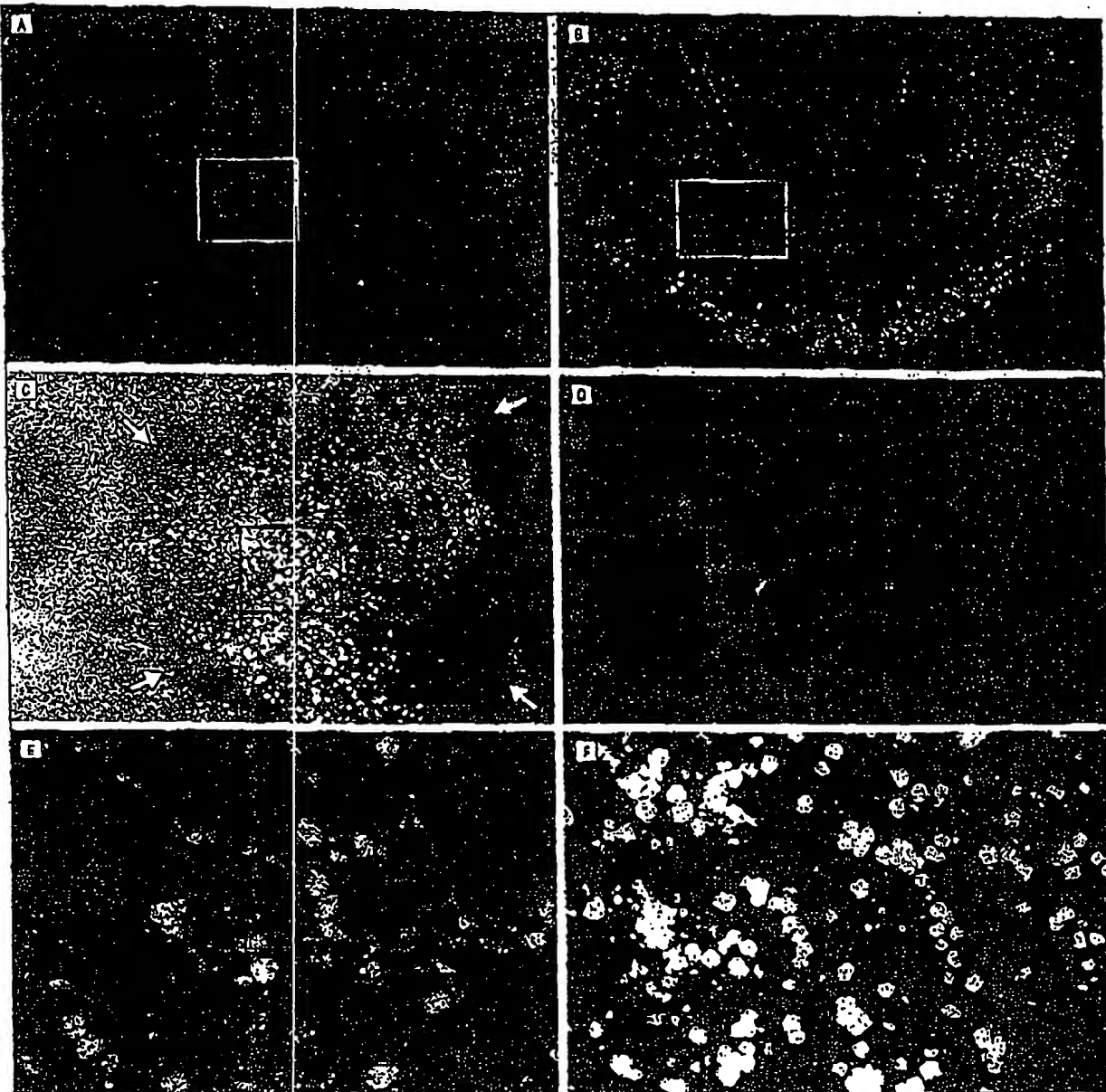


Figure 2. Photographs from fluorescent microscopy of flat mounts of retinal pigment epithelium (RPE)-choroid-scleral complex. A to C, Four, 7, and 14 days after injection, respectively. D, Hexagonal RPE cells expressing green fluorescent protein. E and F, Cellular infiltration and RPE damage in virus-contacted area at 7 (E) and 14 (F) days after injection. Arrows in C point to the virus-contacted area. D, E, and F show higher magnifications of the squares in A, B, and C, respectively (original magnification $\times 10$ [A-C], $\times 50$ [D-F]).

group, but there was no statistical difference when compared with the normal control group ($P=.29$).

WHITE BLOOD CELL AND DIFFERENTIAL COUNTS AND ANIMAL TOLERANCE TO THE IMMUNOSUPPRESSION

To monitor the effect of immunosuppression on systemic immunocompetence, peripheral-blood samples were collected for white blood cell count and differential count (Figure 8). Immunosuppression by cyclosporine plus sirolimus significantly decreased the absolute number of

white blood cells, in comparison with the vehicle-treated group ($P=.02$). White blood cell differentiation showed that the number of lymphocytes and monocytes was significantly reduced ($P<.001$ and $P=.02$, respectively). Cyclosporine alone significantly reduced lymphocytes and monocytes ($P=.02$ and $P=.03$, respectively) but did not affect the absolute number of total white blood cells ($P=.09$). Sirolimus decreased the number of lymphocytes ($P=.02$) but affected neither the number of monocytes nor the total number of white blood cells ($P=.85$ and $P=.43$, respectively). In all 3 groups, neutrophils were not affected ($P>.30$).

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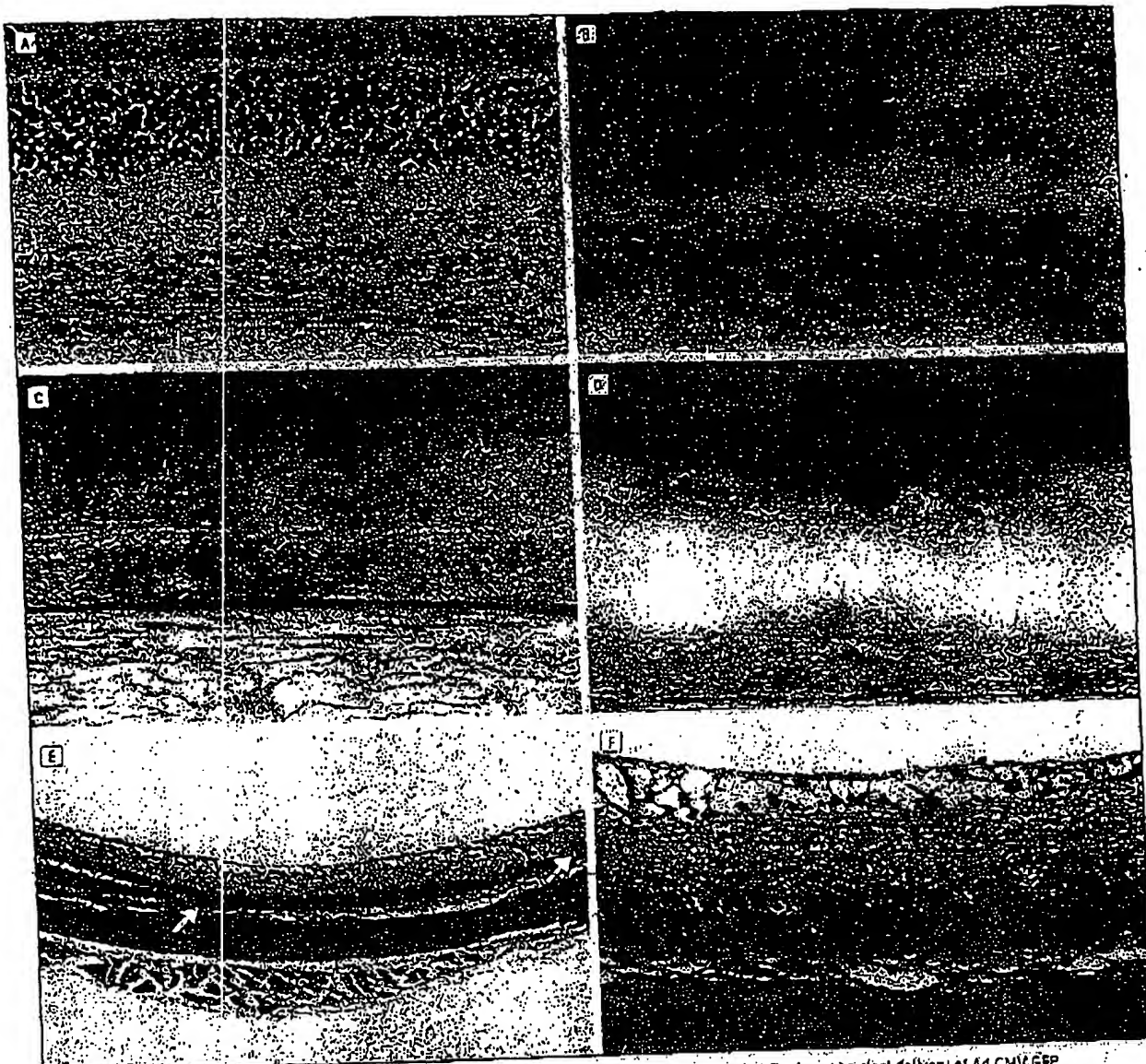


Figure 3. Light micrographs showing cell infiltration (A-D, serial sections) and retinal degeneration (E and F) after subretinal delivery of Ad.CMV.GFP. A, Inflammatory cells in the choroid, subretinal space, and neural retina (ch indicates choroid; pos, photoreceptor outer segments; onl, outer nuclear layer; and inl, inner nuclear layer). B to D, Immunophenotyping for CD8⁺ (B) and CD4⁺ (C) cytotoxic T lymphocytes and CD68⁺ macrophages (D) at 7 days after injection. E and F, Micrographs from a paraffin section showing retinal degeneration at an area between 2 arrows in E, 5 weeks after injection. F is a higher magnification of E (A-D, stained or counterstained with hematoxylin, original magnification $\times 100$; E, hematoxylin-eosin, original magnification $\times 25$; F, hematoxylin-eosin, original magnification $\times 100$).

Weight loss occurred in all animals treated with cyclosporine plus sirolimus and cyclosporine, but not by sirolimus or vehicle (Figure 9). Cyclosporine plus sirolimus- and cyclosporine-treated animals continuously experienced weight loss for 1 to 2 weeks but returned to their baseline weight at 1 week after withdrawal of immunosuppression, and all rats with initial weight loss gained weight beyond their pretreatment levels at 2 weeks after the immunosuppression was suspended.

Biochemical analysis showed that all groups had similar blood glucose levels at 5 weeks after injection (Table 2). However, cyclosporine plus sirolimus and cyclosporine significantly increased the levels of urea and creatinine and

decreased the levels of inorganic phosphate and alkaline phosphatase, but they did not change the levels of alanine aminotransferase and aspartate aminotransferase. Except for a decreased level of alkaline phosphatase, no other biochemical changes were found in the sirolimus-treated group.

By histologic examination, no pathogenicity was found in the livers and kidneys of all groups that had experienced transient immunosuppression (data not shown).

COMMENT

A number of studies have examined rAd-mediated gene transfer after subretinal injection, and the duration of trans-

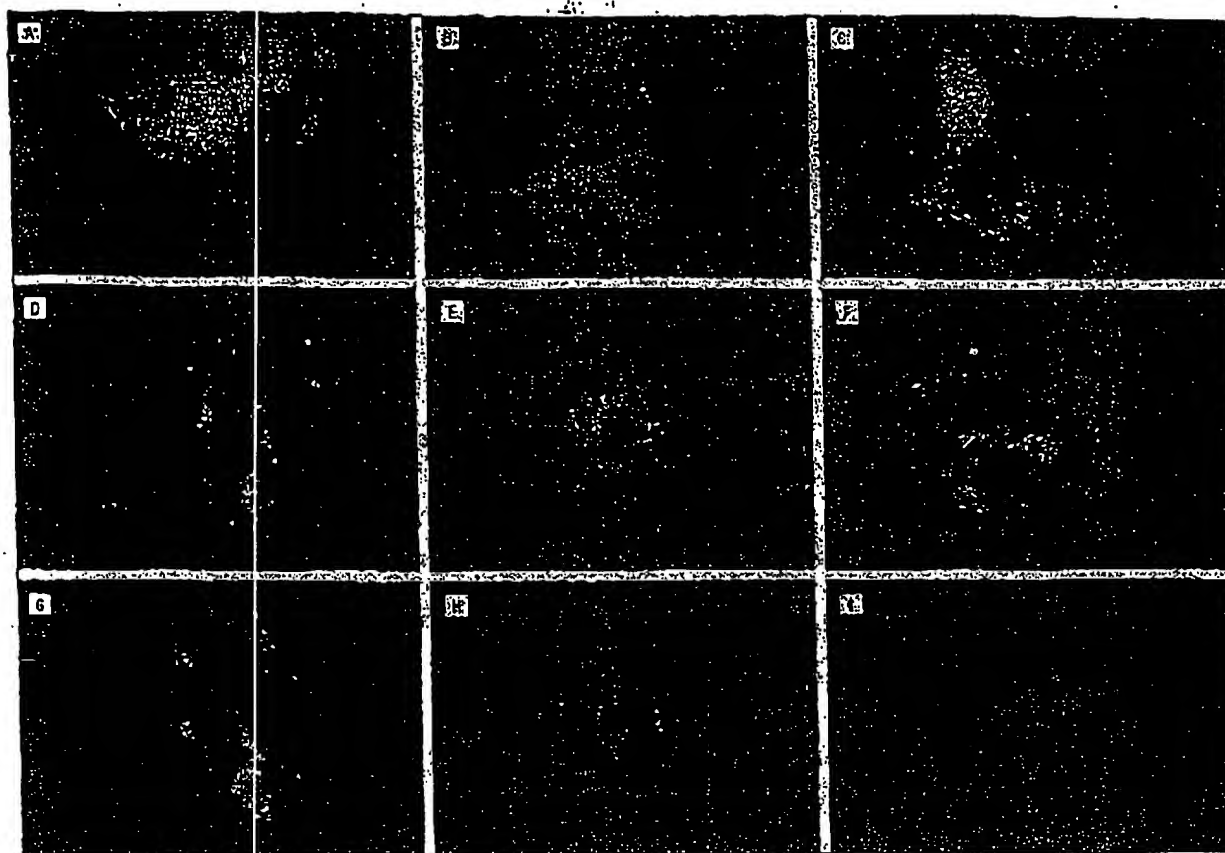


Figure 4. Fluorescent images showing green fluorescent protein expression in animals treated with transient immunosuppression (A, D, and G were treated with cyclosporine and sirolimus; B, E, and H, with cyclosporine; and C, F, and I, with sirolimus), 4 days (A, B, and C), 4 weeks (F), 5 weeks (D, E, and I), 8 weeks (H), and 10 weeks (G) after injection (original magnification $\times 6$).

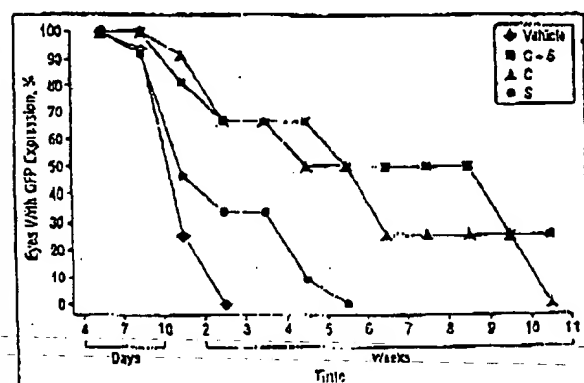


Figure 5. Percentages of eyes with green fluorescent protein (GFP) expression after recombinant adenovirus injection and immunosuppression. The percentage of positive eyes includes any eye expressing GFP signal detected by fundus fluorescein photography. The data represent 10 to 16 eyes per group from 4 days to 5 weeks, and 4 eyes per group from 6 to 10 weeks after injection. C indicates cyclosporine; S, sirolimus.

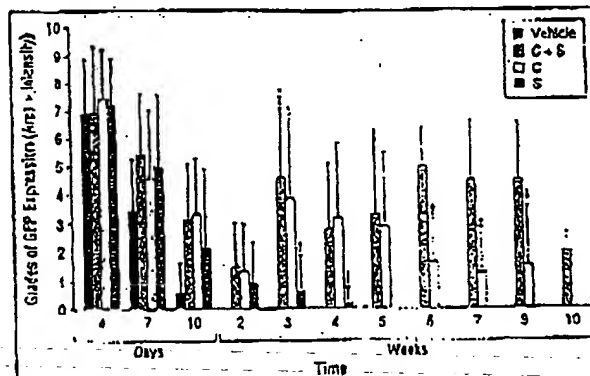


Figure 6. Grades of green fluorescent protein (GFP) expression in animals treated by different strategies of immunosuppression after subretinal injection of recombinant adenovirus. C indicates cyclosporine; S, sirolimus. Asterisk indicates $P < .01$ vs 2 weeks after injection; dagger, $P < .01$ vs group treated with cyclosporine and sirolimus; and double dagger, $P < .05$ vs group treated with cyclosporine and sirolimus; all by unpaired t test.

gene expression varies from 2 weeks to several months.^{24,37} In this study, the length of transgene (GFP) expression was monitored by FFP in real time. We did observe a difference in method sensitivity between FFP in living animals and fluorescent microscopy with R-C-S whole mounts.

By fluorescent microscopy, GFP expression was stronger than that observed by FFP, and in some eyes, GFP expression occasionally lasted longer than observed by FFP. In most eyes, however, we detected little GFP expression more than 2 weeks after injection, indicating short-term

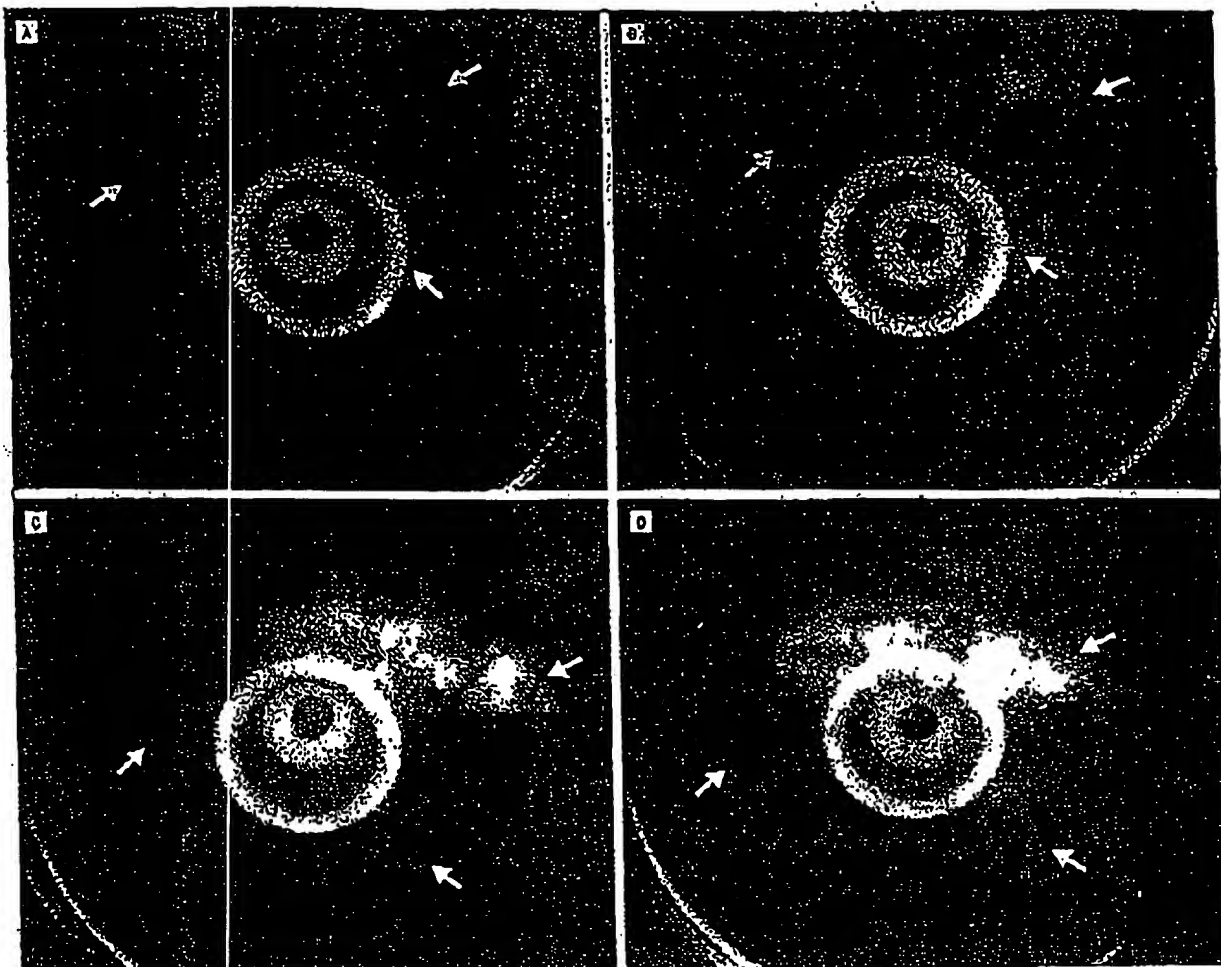


Figure 7. Changes of retinal pigmentation after subretinal injection of recombinant adenovirus, with or without transient immunosuppression (A and B, cyclosporine plus sirolimus; C and D, vehicle), 4 weeks (A and C) and 8 weeks (B and D) after injection. Arrows indicate the disturbed areas (original magnification $\times 6$).

Table 1. Dark-Adapted Electroretinography 4 Weeks After Subretinal Injection of Ad.CMV.GFP With or Without Transient (2 Weeks) Immunosuppression

Group	No.	a-Wave Implicit Time, ms	a-Wave Amplitude, μV	b-Wave Implicit Time, ms	b-Wave Amplitude, μV
Normal (noninjected) control	6	15.83 ± 0.75	190.30 ± 70.07	83.83 ± 3.06	512.17 ± 98.22
Ad.CMV.GFP + vehicle	4	16.75 ± 1.28	175.25 ± 37.29	81.00 ± 8.16	$378.75 \pm 32.04^*$
Ad.CMV.GFP + cyclosporine + sirolimus	5	18.20 ± 1.64	184.00 ± 49.89	67.00 ± 9.03	$442.80 \pm 107.64^*$

* $P < 0.05$ vs normal control group by *t*-test.

rAd-mediated transgene expression. The rAd-induced complications were demonstrated, as cellular infiltration consisted of CD4⁺ and CD8⁺ cytotoxic T lymphocytes and CD68⁺ macrophages, clumped retinal pigmentation, decreased b wave of the ERG, and retinal degeneration on histologic examination. Finally, we have dramatically improved transgene expression through temporary immunosuppression by the combination of 2 synergistic immunosuppressants, cyclosporine and sirolimus.

Traditionally, several sites in the body have been described as having immune privilege, including the

cyc. Earlier reports suggested that the subretinal space is totally immune-privileged with respect to directly delivered rAd^{12,23}; however, recent investigations showed dramatic controversy.^{14,15} In this study, the gradual decrease of rAd-mediated transgene expression could be the result of several changes, such as the cytomegalovirus promoter shutdown and immune responses to the viral vector and GFP protein. We cannot exclude that the reduction of GFP expression may be partially due to inactivation of the cytomegalovirus promoter. However, we found a coincidence of reduction of GFP expression

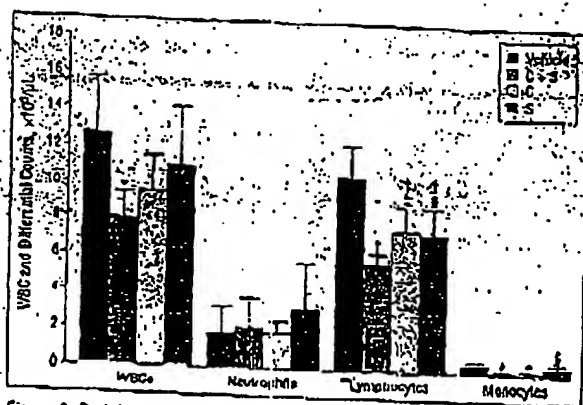


Figure 8. Peripheral white blood cell (WBC) count and differential cell counts after transient (2 weeks) immunosuppression. Blood samples were taken at 5 weeks. C indicates cyclosporine; S, sirolimus. Asterisk indicates $P < 0.05$ vs vehicle treatment; dagger, $P < 0.01$ vs vehicle treatment; double dagger, $P < 0.01$ vs group treated with cyclosporine and sirolimus; and section mark, $P < 0.05$ vs group treated with cyclosporine and sirolimus; all by unpaired t test.

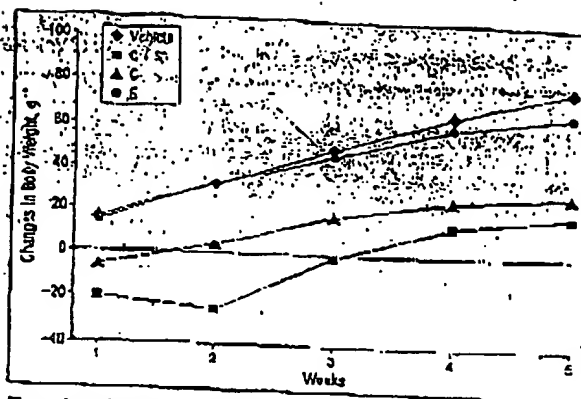


Figure 9. Variation in the weight of rats measured at weekly intervals. The data represent 5 to 8 animals in each group at every time point. The strategies of immunosuppression were performed by daily intraperitoneal injection for 2 weeks. C indicates cyclosporine; S, sirolimus.

with recruitment of inflammatory cells. Immunophenotyping showed that most of the infiltrating cells are CD4⁺ and CD8⁺ cytotoxic T lymphocytes. We further demonstrated that combined immunosuppression by cyclosporine plus sirolimus extended GFP expression by 6-fold. All of these facts directly and indirectly indicate that the decreased GFP expression with time is more likely due to immune responses. It has been recently demonstrated that subretinal readministration of the recombinant adeno-associated virus still resulted in GFP expression.²⁴ In our own hands, recombinant adeno-associated virus-mediated GFP expression remained relatively consistent for up to 18 months.²⁵ These facts indicate that the rAd-induced immune responses are related to the presence of rAd vector rather than GFP protein. Previous studies have demonstrated that the mechanisms involved in elimination of the rAd vector by host responses include (1) innate immunity, (2) major histocompatibility complex class I-restricted cytotoxic T-cell responses, and (3) types 1 and 2 helper T-cell-mediated cellular and humoral immune responses.^{26,27} The reason for the lost immune privilege after subretinal delivery of rAd is unclear. It is highly possible that the current technique of subretinal injection breaks the blood-retinal barrier and allows some rAd particles to leak out of the eye. Once the immune system is primed by the leaked rAd, the immune privilege of the eye is lost.¹⁴ Moreover, administration of a high dose of adenovirus could also evoke a prominent delayed-type hypersensitivity after the second administration of the same adenoviral vector.²⁸ All these pitfalls limit the application of the first generation of rAd for ocular gene therapy.

It is important to use generally accepted clinical techniques to evaluate the intracocular safety when considering rAd for ocular gene therapy, particularly by subretinal injection. In this study, fundus color photography clearly demonstrated a progressive retinal depigmentation in the rAd-injected area. The results of ERG, used to monitor the retinal function, have been reported pre-

viously to be temporarily affected.¹³ The effect of rAd on ERG response seems to be dependent on the rAd concentration. Delivery of 6×10^4 plaque-forming units of rAd intravitreally or subretinally resulted in a reversible decrease in ERG.¹³ In this study, increasing rAd concentration (6×10^7 plaque-forming units) induced a significant decrease in the b-wave amplitude of the dark-adapted ERG at 4 weeks after injection. Our results obtained from fundus color photography, ERG, and histologic examination suggest that subretinal delivery of rAd affects the neural retina function, indicating a potential retinal toxic effect.

The mechanism of retinal depigmentation is not clear. There is evidence suggesting that activated peripheral cytotoxic T cells in response to rAd migrate into the injected site, where they induce apoptosis through a Fas-Fas ligand-dependent mechanism.²⁹ Fas receptor has been detected on the RPE cells, and binding of Fas-Fas ligand initiates a cascade of apoptosis.^{30,31} This theory may also explain our later results showing that the combined immunosuppression improved GFP expression but was unable to stop the progress of retinal damage. Other possible mechanisms by which cytotoxic T cells kill the RPE cells involve perforin-granzyme and tumor necrosis factor interactions.³² It is also possible that the delivered rAd induces proinflammatory cytokine release from RPE or photoreceptors and that they have pathologic effects even before the appearance of inflammatory cells.³³ Moreover, the rAd-induced RPE damage can further result in atrophy of the choroidal capillary beds.³⁴ All these alterations will affect transportation of nutrients from the choroid to the neural retina, which further leads to the irreversible retinal degeneration. Once the retina is degenerated, the damage would be permanent.

Several approaches have been taken to circumvent the immune responses to adenoviral vectors, such as deletion of several regions from the adenoviral genome to engineer a less immunogenic vector. Unfortunately, further deletion of the adenoviral genome makes the virus less efficient or more difficult to propagate and obtain in high titer.^{35,36} The immune responses can be avoided provided that the vectors are delivered into neonatal ani-

Table 2. Blood Analysis		Alkaline phosphatase (ALP)				Alanine aminotransferase (ALT)				Aspartate aminotransferase (AST)			
Group	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle
ALP (U/L)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
ALT (U/L)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
AST (U/L)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

*Blood samples were collected 5 weeks after viral injection. ALP indicates alkaline phosphatase; ALT, alanine aminotransferase; and AST, aspartate aminotransferase.

†To convert to millimoles per liter, multiply by 0.0555.

‡To convert to micromoles per liter, multiply by 0.1667.

§To convert to micromoles per liter, multiply by 88.4.

||To convert to micromoles per liter, multiply by 0.1053.

¶P<0.05 vs vehicle-treated group by t test.

mals, which have an immature immune system,³⁷ but this strategy is not of clinical relevance to ocular gene therapy.

Suppression of the host immune system by immunosuppressants such as cyclosporine, tacrolimus, and etoposide has significantly improved the efficacy of transgene expression.^{38,39} However, most of the currently available immunosuppressants display a narrow range between efficacy and toxic side effects, making them less attractive for clinical application.^{16,17} One strategy to overcome this limitation is to combine low doses of synergistic drugs to achieve more therapeutic effect. In this study, we have demonstrated that transient treatment with cyclosporine and sirolimus significantly prolongs rAd-mediated transgene expression, and the combination is more effective than single-drug application. From the results of white blood cell counting and differential counting, we showed that cyclosporine and sirolimus, either alone or in combination, dramatically decrease the number of lymphocytes and/or monocytes in serum, with the combination being the most effective. These results indicate that transient treatment with cyclosporine and sirolimus resulted in systemically cellular immunosuppression that is sufficient to prolong transgene expression.

Although we could not conclude whether the combined effect is synergistic or additive, a number of previous studies have demonstrated that cyclosporine plus sirolimus acts synergistically on immunosuppression.^{20,21} In the eye, cyclosporine and sirolimus inhibited retinal S-antigen-primed lymphocyte proliferation, and they showed a marked synergistic effect over a wide dose range as determined by a median-effect analysis.²⁰ The synergistic effect of cyclosporine and sirolimus calculated in vitro was further confirmed in vivo in the treatment of experimental autoimmune uveoretinitis, demonstrated as complete inhibition of disease in all animals treated with the combination regimen.²⁰ The synergistic effect of cyclosporine and sirolimus allows the reduced doses of each drug to improve rAd-mediated transgene expression.

Interestingly, we observed rebound GFP expression in the groups treated with cyclosporine plus sirolimus and with cyclosporine but not in the sirolimus-treated group at 1 week after withdrawal of immunosuppression. Peripheral white blood cell counts and differential counts suggest that strong immunosuppression was achieved by cyclosporine plus sirolimus and by cyclosporine. It is possible

that the strong effect of cyclosporine plus sirolimus and cyclosporine during the first 2 weeks not only circumvents the systemic immunity but also suppresses cell activity. There is also another possibility, that continuous immunosuppression by cyclosporine plus sirolimus and cyclosporine may inhibit the activity of the cytomegalovirus promoter in certain contents, and withdrawal of immunosuppression could relieve this inhibition.

Finally, some weight loss occurred after the combined immunosuppression, but the weight was quickly regained after withdrawal of the treatment. Although biochemical analysis of serum showed some changes in renal and liver function, histologic examination did not support obvious damage.

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Corresponding author and reprints: Pirosha E. Rakoczy, PhD, Department of Molecular Ophthalmology, Lions Eye Institute, 2 Verdun St, Nedlands, Perth, Western Australia 6009, Australia (e-mail: rakoczy@eyllene.uwa.edu.au).

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